SPECIFICATION

Novel Collectin

[Field of the Invention]

The present invention relates to a novel collectin which is useful for investigating mechanisms of biological defense, and is expected to be applied for utilizing as materials for medicines because it may have physiological activities including anti-viral activities and the like.

[Background Art]

Collectin is a generic name of proteins having calcium-dependent carbohydrate recognition domain (CRD) and collagen-like region, and the member of these proteins is conceived to involve in basic immunity systems against a wide spectrum of microorganisms such as bacteria and viruses.

The collectins which have been identified heretofore include mannan-binding protein ~~(MBP)~~ (MBP, SEQ ID NO:27), surfactant protein A ~~(SP-A)~~ (SP-A, SEQ ID NO:28), surfactant protein D ~~(SP-D)~~ (SP-D, SEQ ID NO:29) and conglutinin. These collectins are known to be constituted from basic structures comprising four unique regions of: (1) calcium-dependent carbohydrate recognition domain (CRD), (2) neck region, (3) collagen-like region and (4) N-terminal region containing cysteine [Malhortra *et al.*, *Eur.J.Immunol.* Vol.22, 1437-1445, 1992] (see, Fig. 1(a)). A subunit can be formed from the three basic structures through making a triple helix in the collagen-like region, and such subunit constitutes an oligomer, e.g., trimer, tetramer and hexamer.

In vertebrates, mechanisms involving cellular immune responses and specific antibody reactions are considered as dominant host-defense systems against inversion of the pathogenic bacteria, viruses and the like. Recently,

involvement in nonspecific immune responses of the lectins such as conglutinin has been suggested, for example, it was reported that the lectins may play important roles in neutralizing and removing the various microorganisms in infants having insufficient maternal antibodies and undeveloped specific defense systems [Super *et al.*, *Lancet*, Vol.2, 1236-1239, 1989].

Moreover, with respect to the roles of the lectins in the biological host-defense systems, it was reported that the host becomes susceptible to infection by, for example, a reduction of the MBP concentration in blood due to genetic mutation of the MBP gene [Sumiya *et al.*, *Lancet*, Vol.337, 1569-1570, 1991].

The present inventors have found that the conglutinin and the mannan-binding protein can inhibit infection and hemagglutination activity of H1 and H3 Type Influenza A viruses [Wakamiya et al., *Glycoconjugate J.*, Vol.8, 235, 1991; Wakamiya *et al.*, *Biochem. Biophys. Res. Comm.*, Vol.187, 1270-1278, 1992].

Thereafter, the present inventors isolated a cDNA clone encoding the conglutinin, and found that closer correlation may exist between the conglutinin gene and various surfactant protein D gene [Suzuki *et al.*, *Biochem. Biophys. Res. Comm.*, Vol.191, 335-342, 1993].

As described above, the collectin has been expected to be useful in investigating mechanisms of biological defense, and be applicable for utilizing as materials for medicines, however, the presence of any other molecular species belonging to this protein family has not been elucidated.

[Disclosure of the Invention]

The present invention was accomplished in consideration of the aforementioned state of art, and is directed to provide a novel collectin which can be expected to exhibit physiological activities such as anti-bacterial, anti-viral activity, especially in human body.

Accordingly, to provide the following polynucleotide and protein which share characteristic structures of those belonging to the collectin family, and which are distinct from the collectins reported heretofore is intended by the present invention:

[1] A polynucleotide comprising the nucleotide sequence which encodes a protein having the amino acid sequence set out in SEQ ID NO: 2;

[2] A polynucleotide comprising the nucleotide sequence set out in SEQ ID NO: 1;

[3] A polynucleotide which encodes a collectin protein, wherein said polynucleotide can hybridize under a stringent condition with a probe produced from a genomic clone which shares high homology to a consensus collectin amino acid sequence set out in SEQ ID NO:3
Glu-Lys-Cys-Val-Glu-Met-Tyr-Thr-Asp-Gly-Lys-Trp-Asn-Asp-Arg-Asn-Cys-Leu-Gln-Ser-Arg-Leu-Ala-Ile-Cys-Glu-Phe;

[4] A polynucleotide which can hybridize with any of the polynucleotide according to any of [1] to [3], wherein the protein encoded by said polynucleotide comprises: (1) Ca^{2+}-dependent carbohydrate recognition domain (CRD), (2)neck region, (3) collagen-like region, and (4) N-terminal region containing cysteine;

[5] A collectin protein encoded by the polynucleotide according to any of [3] or [4];

[6] A collectin protein comprising the amino acid sequence set out in SEQ ID NO:2;

[7] A collectin protein comprising the amino acid sequence encoded by

the polynucleotide comprising the nucleotide sequence set out in SEQ ID NO: 1;

[8] The collectin protein according to any of [5] to [7], wherein the amino acid sequence of the protein comprises deletion, substitution and/or addition of one or more amino acids, and wherein the protein comprises: (1) Ca^{2+}-dependent carbohydrate recognition domain (CRD), (2) neck region, (3) collagen-like region, and (4) N-terminal region containing cysteine.

[Brief Description of the Drawings]

Figure 1 is a profile showing basic structures and overviews of the principal collectins reported heretofore;

Figure 2 shows the alignment of the preceding half portions of amino acid sequences (SEQ ID NOS: 27-29) of three collectins reported heretofore;

Figure 3 shows the alignment of the latter half portions of the amino acid sequences (SEQ ID NOS: 27-29) in Figure 2;

Figure 4 shows each of the primers used for sequencing the novel collectin polynucleotide of the present invention, and maps of the nucleotide sequence which were read from the sequencer (a, b) ~~(a, jb)~~; and an ORF of the obtained collectin (a);

Figure 5 shows the alignment of the preceding half portions of amino acid sequences (SEQ ID NOS: 27-29) of the three collectins reported heretofore and the novel collectin of the present invention;

Figure 6 shows the alignment of the latter half portions of the amino acid sequences (SEQ ID NOS: 27-29) in Figure 5;

Figure 7 illustrates a comparison of the basic structures of the three collectins reported heretofore and the novel collectin of the present invention comprising (1) Ca^{2+}-dependent carbohydrate recognition domain (CRD), (2)neck region, (3) collagen-like region, and (4) N-terminal region containing cysteine;

Figure 8 shows a result of genomic Southern analysis with the novel

collectin of the present invention;

Figure 9 shows a result of genomic Northern analysis of various human tissues, i.e., (a) heart, (b) brain, (c) placenta, (d) lung, (e) liver, (f) skeletal muscle, (g) kidney and (h) pancreas with the novel collectin of the present invention to clarify the tissue distribution of the collectin;

Figure 10 shows a result of genomic Southern analysis of genes from various vertebrates, i.e., (a) human, (b) monkey, (c) rat, (d) mouse, (e) dog, (f) cow, (g) rabbit and (h) chicken with the novel collectin of the present invention to elucidate the conservation of the collectin during the species; and

Figure 11 shows a phylogenetic tree of various collectins.

[Best Mode for Carrying Out the Invention]

In the preferred embodiment of the present invention, the probe in the above [3] may be amplification products by PCR which was performed using the primers which have the following sequences:

TTTTGATGGAGGCTCCATACC (SEQ ID NO: 7); and

CTGCCAACACACTCATCGCTG (SEQ ID NO: 8).

Thus, desired polynucleotide encoding the collectin protein can be suitably obtained.

Moreover, in the preferred embodiment, the polynucleotide may be cDNA.

Further, the protein of the present invention may preferably consist of the amino acid sequence which is identical to that derived from human, because it can be expected to exhibit physiological activities such as anti-bacterial, anti-viral activity in human body, thereby useful physiologically active material for medicines may be resulted. Therefore, it is intended that the protein of the present invention may be the collectin protein derived from

human. We examined various human tissues, and expression of the collectin protein in human liver which seems to be useful could be revealed.

The stringent hybridization condition in the inventions of the above [3] and [4] may include for example, a series of the following steps for the hybridization: prehybridization in a solution of 5 x SSC (prepared by diluting 20 x SSC (3 M NaCl, 0.3 M sodium citrate)), 1% blocking agent (Boehringer Mannheim), 0.1% N-lauroyl sarcosine, and 0.02% SDS, at 68℃ for an hour; and hybridization in a solution of 5 x SSC, 1% blocking agent, 0.1% N-lauroyl sarcosine, and 0.02% SDS containing cDNA probes (10 ng/ml), at 55℃ for 16 hours; washing in a solution of 2 x SSC/0.1% SDS for 5 minutes, 2 times; and washing in a solution of 0.5 x SSC/0.1% SDS at 55℃ for 15 minutes, 2 times. However, several modifications/alterations of these conditions may be made, based on the knowledge of the skilled art, such as the concentration of the solution, incubation temperature and time.

In addition, the N-terminal region containing cysteine in the above [8] may contain at least one cysteine residue, preferably one cysteine residue.

Further, deletion, substitution and/or addition of one or more amino acids in the above [8] may be those which result in less changes of hydrophilic/hydrophobic nature, acidic/basic nature, and constitutional residues of the collectin proteins, without bringing much alterations of the properties in the above-described four regions, specifically in both of the regions: (1) Ca^{2+}-dependent carbohydrate recognition domain (CRD) and (3) collagen-like region. Taking the structural sequences of the proteins belonging to the collectin family reported heretofore into account, for example, deletion, substitution and/or addition of 1-10 amino acid residues in (1) Ca^{2+}-dependent carbohydrate recognition domain (CRD) and in (2)neck region, 1-100, preferably 1-15 amino acid residues in (3) collagen-like region, and 1-20 amino acid residues in (4) N-terminal region containing cysteine and

the signal sequence may be allowed.

The present invention will be described in more detail by the non-limiting illustrative examples. It is intended that the present invention encompasses all modifications and variations which occur to those skilled in the art upon consideration of the disclosures herein, and in particular those embodiments which are within the broadest proper interpretation of the claims and their requirements.

The Examples illustrate: the search on EST data base (Example 1); preparation of the probes for screening (Example 2); screening of cDNA library derived from human liver (Example 3); sequencing of nucleotide sequence of the novel collectin (Example 4); genomic Southern analysis of the novel collectin (Example 5); Northern analysis of the novel collectin with various human tissues (Example 6); genomic Southern analysis of the novel collectin with tissues from various species of animals (Example 7); and genetic study of the novel collectin (Example 8).

Example 1: Search on EST Data Base

Highly conserved regions between molecules of known collectin proteins, i.e., MBP, SP-A and SP-D were searched by comparing the amino acid sequences thereof (see Figures 2 and 3, in which amino acid residues which were recognized to be homologous between those proteins were boxed). As a result, it was suggested that the region consisting of 27 amino acids, namely from amino acid 220 to 246 of human MBP sequence (shown in Figure 3, reversed characters), was highly homologous. Therefore, some consensus sequences corresponding to this region were prepared, and conducted searches on EST (Expressed Sequence Tags) data base with such sequences. For this search, the EST data base published on October 11, 1996 was employed, which included 676750 sequences.

Consequently, some data comprising homologous amino acid sequences were obtained. Searches on GenBank/EST data base with thus obtained data of the amino acid sequences were further conducted, and deduced whether they were derived from known, or unknown substances. Thereby, it was confirmed that data including highly homologous but unknown nucleotide sequence (registered as: R29493) could be identified when the following amino acid sequence was used as a consensus sequence:

Glu-Lys-Cys-Val-Glu-Met-Tyr-Thr-Asp-Gly-Lys-Trp-Asn-Asp-Arg-Asn-Cys-Leu-Gln-Ser-Arg-Leu-Ala-Ile-Cys-Glu-Phe (SEQ ID NO: 3).

The data contained the sequence of 5'-terminal 326 nucleotides of a clone F1-1006D from human embryonic (22 weeks old) liver cDNA library.

Thereafter, the clone was kindly provided from the owner, Mr. Hee-Sup Shin (Pohang Institute of Science & Technology (Pohang, Korea)). The insert size of the clone was about 600 bp, with the 5'-terminal end being incorporated adjacent to the nucleotide sequence set out in SEQ ID NO: 4, while 3'-terminal end being incorporated into plasmid pSK(-) (pBluescriptIISK(-)) at XhoI restriction site.

Example 2: Preparation of the Probes for Screening

The insert of the clone described above in Example 1 was excised using EcoRI and XhoI, then incorporated into pUC18, and sequenced using a primer (Pharmacia, M13 Universal Primer (SEQ ID NO: 5, 5'-fluorescein-CGACGTTGTAAAACGACGGCCAGT-3')) and M13 Reverse Primer (SEQ ID NO: 6, 5'-fluorescein-CAGGAAACAGCTATGAC-3').

In the nucleotide sequence obtained, an open reading flame was selected through matching it to the collectin amino acid sequence. The nucleotide sequence corresponding to the amino acid sequence which could be read from the above open reading flame was picked out, and primers for

digoxigenin (DIG) labeled cDNA probes (Reverse Primer (SEQ ID NO: 7) and Forward Primer (SEQ ID NO: 8)) corresponding to the parts of the nucleotide sequences were produced using DNA/RNA Synthesizer (Applied Biosystems, 392A). DIG labeling was achieved using PCR DIG Probe Synthesis Kit (Boehringer Mannheim). The reaction mixture contained: DNA fragments which were the excised inserts from the clone F1-1006D with EcoRI and XhoI (4.4 ng/μl, 12 μl: 52.8 ng), 10 x buffer: 5 μl, 25 mM $MgCl_2$: 5 μl, dNTP (PCR Labeling Mix): 2.5 μl, 20 μM Reverse Primer : 2.5 μl, 20 μM Forward Primer : 5 μl, H_2O : 18 μl, Taq Polymerase : 0.5 μl. PCR reaction was carried out using Zymoreactor (Atto Corp.,) through 35 cycles of: 1 minute at 92℃, 1minute at 55℃, and 2 minutes at 72℃.

Example 3: Screening of cDNA Library Derived from Human Liver

First, phage cDNA library was titrated as follows. Escherichia coli Y1090r$^-$ which had been cultured at 37℃ for 16 hours in mLB medium (LB medium (1 g trypton, 0.5g yeast extract and 0.5 g NaCl in total volume of 100 ml) containing 10 mM $MgSO_4$ and 0.2% maltose), 0.2 ml, and 0.1 ml of cDNA library serially diluted with SM buffer (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 2 M Tris-HCl (pH 7.5) 25 ml, and 2% gelatin 5 ml in total volume of 1L) were incubated at 37℃ for 15 minutes, then the mixtures were added to 2.5 ml of LB-TOP agar (0.75% agar/LB medium) to make homogenous solutions, and plated onto LB Plates (Iwaki Glass, 90 mm ϕ , 1.5% agar/LB medium). The added solutions were hardened at a room temperature for 15 minutes, then incubated for 5 hours at 42℃. The plaques on each of the plates were counted, and the titer of the phage was calculated. The titer calculated hereby was 2.3×10^{10} pfu/ml.

The screening with thus titrated cDNA library using the probes prepared in Example 2 was conducted as follows. Escherichia coli Y1090r$^-$ which had been cultured at 37℃ for 16 hours in mLB medium, 0.6 ml and cDNA library diluted with SM buffer to 1×10^5 pfu were incubated at 37℃

for 15 minutes, then the mixture was added to 7.5 ml of LB-TOP agar (0.75% agarose) to make a homogenous solution. The solution was plated onto ten LB square plates of 140 mm^2 (Nissui Seiyaku), hardened at a room temperature for 15 minutes, then the plates were incubated for 5 hours at 42℃. After plaque formation of each of the plates was determined, the transfer to the nylon membranes, using Nytran 13N (Schleicher and Schuell Co.) as a filter was performed. The filters (12.5 cm x 9.0 cm in size) were immersed in distilled water for 10 minutes to be wet, then the excess water was removed on Whatmann 3MM Paper. The filters were placed on the plates having the plaques formed thereon. After standing for two minutes, the filters were recovered and air dried for 10 minutes. The phage DNA on the filters was denatured for 2 minutes with 0.2 M NaOH/1.5 M NaCl, followed by neutralization with 0.4 M Tris-HCl (pH7.6) / 2 x SSC for 2 minutes and washing with 2 x SSC for 2 minutes. Thereafter, the DNA was fixed on the membrane by UV irradiation with GS GENE LINKER (BioRad).

Hybridization and detection of the signals were conducted as follows. The filters were soaked in 2 x SSC, and the excess water was removed using Whatmann 3MM Paper. Then, the filters were placed in a hybridization bag and prehybridization at 68℃ for one hour in a hybridization solution (5 x SSC, 1% blocking agent, 0.1% N-lauroyl sarcosine and 0.02% SDS) was performed. Subsequently, the hybridization solution was removed from the bag, and the hybridization solution containing DIG labeled cDNA probe at a concentration of 10 ng/ml was added thereto, and hybridization was proceeded at 55℃ for 16 hours. After the hybridization was completed, the filters were washed in a solution of 2 x SSC/0.1% SDS at a room temperature for 5 minutes, 2 times; and further washed in a solution of 0.5 x SSC/0.1% SDS for 15 minutes at 55℃, 2 times. Then, SDS was removed using DIG buffer I (100 mM Tris-HCl, 150 mM NaCl (pH7.5)) for 1 minute, and the filters were blocked with DIG buffer II (1 % blocking agent in DIG buffer I)

for 30 minutes. After washing the filters with DIG buffer I for one minute, a solution of alkaline phosphatase labeled anti-DIG antibody (Boehringer Mannheim) which was diluted to 5000-fold in DIG buffer II was added, and the reactions between antigen and antibody were allowed for 30 minutes. The filters were then washed twice with DIG buffer I for 15 minutes at a room temperature. Through the subsequent treatment of the filters with DIG buffer III (100 mM Tris-HCl, 100 mM NaCl (pH 9.5), 50 mM $MgCl_2$) for 3 minutes, the concentration of Mg^{2+} was elevated. Finally, a solution of NBT/BCIP (WAKO Chem., Co.) in DIG buffer III was added for color development, thereby 13 positive clones were identified.

The plaques corresponding to these clones were excised from the plates and placed in the tubes containing 1 ml of SM buffer. After stirring for 10 minutes, each of the buffer solution was serially diluted with SM buffer, and 0.1 ml of the diluted solution was mixed with 0.2 ml cultures of Escherichia coli Y1090r$^-$ which had been cultured in mLB medium for 16 hours at 37℃. The mixture was incubated for 15 minutes at 37℃, and added to 2.5 ml of LB-TOP agarose to make a homogenous solution. The solution was plated onto ten LB plates (90mm ϕ), hardened at a room temperature for 15 minutes, then the plates were incubated for 5 hours at 42℃. With respect to several plaques thus obtained, the secondary screening was performed essentially in accordance with the procedures of the primary screening as described above.

Example 4: Sequencing of the Novel Collectin Nucleotide

The plaques from suitable two clones (HLI1-3M and HLI1-9) which were selected from the positive clones obtained in the above secondary screening were excised from the plates, and placed respectively in the tubes containing 1 ml of SM buffer. After stirring, 50 μl of each of the solution was added to 4.95 ml of mLB medium with 50. μl cultures of Escherichia

coli Y1090r- which had been cultured in mLB medium for 16 hours at 37℃. The mixture was cultured for 16 hours at 37℃, then one drop of chloroform was added thereto. After stirring for 3 minutes, the mixture was subjected to centrifuge at 10,000 rpm for 5 minutes to obtain a supernatant therefrom.

The insert DNA was amplified by PCR with TaKaRa LA PCR Kit Ver.2 (TAKARA Syuzo, Co.) using the resulting supernatant as a template. PCR reactions contained the supernatant: 11 μl, 10 x LA PCR Buffer II (Mg^{2+}free): 2.5 μl, 25 mM $MgCl_2$: 5 μl, dNTP Mix: 8 μl, 20 μM λgt11 Reverse Primer (SEQ ID NO: 9: 5'-TTGACACCAGACCAACTGGTAATG-3'): 2.5 μl, 20 μM λgt11 Forward Primer (SEQ ID NO: 10: 5'-GGTGGCGACGACTCCTGGAGCCCG-3'): 1 μl, LA Taq polymerase: 0.5 μl, and H_2O : to make final volume 50 μl. The PCR reaction was performed using Applied Biosystems Gene Amp PCR System 9600, with 30 cycles of: 10 seconds at 98℃, and 5 minutes at 68℃. The PCR product was verified by the electrophoresis with 1% agarose gel, and purified through excising from the gel. For this purification step, Sephaglas BandPrep Kit (Pharmacia) was used.

The excised DNA fragment was incorporated into pCR2.1 vector (Invitrogen, TA Cloning Kit). The recombinant vector was transformed into TOP10F' cell included in the Invitrogen TA Cloning Kit. The transformants were cultured in LB medium (containing 100 μg/ml ampicillin), and two plasmid for each of the clones (HLI1-3M-1, HLI1-3M-2, HLI1-9-1 and HLI1-9-2) were extracted by alkaline SDS method followed by nucleotide sequencing with Autoread Sequencing Kit (Pharmacia) and A.L.F. Autosequencer. M13 Universal Primer (SEQ ID NO: 5) and M13 Reverse Primer (SEQ ID NO: 6) from the Autoread Sequencing Kit were used first, then full length nucleotide sequence was determined based on the resulting elucidated nucleotide sequences using the following primers (3MUO-9R3)

which were produced on a DNA/RNA synthesizer and labeled with FITC (Pharmacia, Fluore Prime):

3MU0: 5'-fluorescein-TAATGGTAGCGACCGGCGCT-3' (SEQ ID NO: 11),
3MU1: 5'-fluorescein-AAACCAATTTATACTCCTGG-3' (SEQ ID NO: 12),
3MU2: 5'-fluorescein-AATATTGGCAAGACTGGGCC-3' (SEQ ID NO: 13),
3MR1: 5'-fluorescein-GATGAGTGTGTTGGCAGCAT-3' (SEQ ID NO: 14),
3MR2: 5'-fluorescein-GTATCTTCCACAATCACAGA-3' (SEQ ID NO: 15),
3MR3: 5'-fluorescein-TTAATTCCTTTCGGCCCCAT-3' (SEQ ID NO: 16),
3MR4: 5'-fluorescein-GCAAAAGAAATAGTACCAGG-3' (SEQ ID NO: 17),
3MR5: 5'-fluorescein-CATATCACCCAGTTCTCCTT-3' (SEQ ID NO: 18),
9U1 : 5'-fluorescein-AGCAGGGATTAGGGAAACTG-3' (SEQ ID NO: 19),
9U3 : 5'-fluorescein-CTGTGAGCGTCATTACAGTT-3' (SEQ ID NO: 20),
9U4 : 5'-fluorescein-GGTTGTCTATATGTCAAATG-3' (SEQ ID NO: 21),
9U5 : 5'-fluorescein-TATGGCCATGGCTATACTTG-3' (SEQ ID NO: 22),
7U3 : 5'-fluorescein-ATCGCTGACTATGTTGCCAA-3' (SEQ ID NO: 23),
9R1 : 5'-fluorescein-CAAGTATAGCCATGGCCATA-3' (SEQ ID NO: 24),
9R2 : 5'-fluorescein-AACTGTAATGACGCTCACAG-3' (SEQ ID NO: 25), and
9R3 : 5'-fluorescein-CATTTGACATATGAACAACC-3' (SEQ ID NO: 26)

As a result, the obtained cDNA clone contained 1295 bases set out in SEQ ID NO: 1, which comprises ORF (open reading frame) of 831 base pairs encoding 277 amino acids as shown in SEQ ID NO: 2.

The outline of this nucleotide sequencing strategy is shown in Figure 4. An ORF of the obtained collectin is illustrated in Figure 4 (a), wherein G-X-Y denotes a collagen-like region. Further, in Figure 4 (b), each of the primer names and maps of the nucleotide sequence which were read from the sequencer (shown as allows), as well as M13 Universal Primer (shown as U)

and M13 Reverse Primer (shown as R) are illustrated.

Figures 5 and 6 show the alignment of the amino acid sequence of the novel collectin of the present invention and those of three collectin proteins reported heretofore. Similarly to Figures 2 and 3, amino acid residues which were recognized to be homologous were boxed.

Furthermore, under structural studies of the sequence of this novel collectin protein, as shown schematically in Figure 7, it was suggested that this protein comprised: (a) N-terminal region containing cysteine, (b) collagen-like region, (c)neck region and (d) carbohydrate recognition domain, as in the case with the known collectins.

However, homology search results on GenBank data base of the DNA and amino acid sequence indicated that the sequence of the obtained protein is distinct from those of the collectins identified previously and it derives from a novel collectin.

Example 5: Genomic Southern Analysis of the Novel Collectin

Genomic Southern analysis was performed in order to clarify whether the novel collectin gene comprising the cDNA sequence shown in Example 4 was a single copy gene or a multi copy gene.

Four μ g of genomic DNA extracted from placenta was digested with a restriction enzyme, EcoRI, HindIII, BamHI, XbaI or SacI, followed by electrophoresis with 0.7% agarose gel at 100 mA, for 3 hours. After the electrophoresis was completed, the DNA was transferred to a nylon membrane (Nytran 13N) to prepare a membrane for the analysis.

For the transfer step, the electrophoresed gel was immersed in 100 ml

of 0.25 N HCl for 10 minutes, washed three times with distilled water, then immersed twice in 100 ml of a denaturalizing solution (1.5 M NaCl, 0.5 M NaOH) for 15 minutes, and immersed in 100 ml of a neutralizing solution (0.5 M Tris-HCl, 3 M NaCl (pH 6.8)) for 30 minutes so that the depurination, denaturalization and neutralization were accomplished. Thereafter, the DNA was transferred using Vacuum Blotting System (Toyobo Engineering, VB-30). In this step, the membrane which had been pretreated by immersing in 2 x SSC for 5 minutes and in 20 x SSC for 5 minutes was used, with a pad which had been soaked with 20 x SSC. After the transfer was terminated, fixation of the DNA was performed by UV irradiation.

Hybridization probe employed for the Southern analysis was the DIG labeled DNA probe corresponding to the cDNA sequence of ORF of the novel collectin as obtained in the above Example 4, which was labeled using the above-described PCR DIG Probe Synthesis Kit. Prior to hybridization, the probe was boiled for 10 minutes, and rapidly frozen with dry ice/ethanol for 5 minutes.

First, the membrane which was subjected to the transfer was immersed in 2 x SSC for 5 minutes, then prehybridization was performed in ExpressHyb Hybridization Solution (Clonetech), 10 ml at 68℃ for 30 minutes. Subsequently, the above frozen probe was diluted to 10 ng/ml in ExpressHyb Hybridization Solution and 2 ml of this solution was used for hybridization at 68℃ for one hour.

Following hybridization, the membrane was washed by shaking: two times in 20 ml of 2 x SSC, 0.1%SDS at a room temperature for 5 minutes and then two times in 20 ml of 0.2 x SSC, 0.1%SDS at 68℃ for 15 minutes. Next, the membrane was washed two times with 50 ml of DIG buffer I (100 mM Tris-HCl, 150 mM NaCl (pH 7.5)) at a room temperature for one minute

in order to remove SDS, and was blocked in DIG buffer II' (1.5% blocking agent in DIG buffer I), 50 ml at a room temperature for one hour. Thereafter, the membrane was treated with 10 ml of alkaline phosphatase labeled anti-DIG antibody which was diluted to 5000-fold in DIG buffer I containing 0.2% Tween20 for 30 minutes followed by washing two times by shaking in 50 ml of DIG buffer I which contains 0.2% Tween20 at a room temperature for 20 minutes. After soaking the membrane twice in 10 ml of DIG buffer III at a room temperature for 3 minutes, it was placed in a hybridization bag, and CSPD (registered trade name, Boehringer Mannheim: chemiluminescence substrate) which was diluted to 100-fold in DIG buffer III was added thereto so that the solution can spread over the membrane. Subsequently, the membrane was exposed to Instant Film 57 (Polaroid).

Consequently, it was speculated that the gene of the obtained novel collectin has been a single copy gene, because only one or two signals could be detected from the respective genomic DNA which was digested with each of the restriction enzymes, as shown in the lanes in Figure 8.

Example 6: Northern Analysis of the Novel Collectin with Various Human Tissues

In order to examine the expression of the mRNA of the novel collectin of the present invention in various human tissues, analysis by Northern hybridization was performed.

Hybridization probe employed for this analysis was the DIG labeled RNA probe corresponding to the cDNA sequence of ORF of the novel collectin (SEQ ID NO: 1), which was labeled using DIG RNA Lasbeling Kit (SP6/T7, Boehringer Mannheim). The analyzed membrane was Human Multiple Tissue Northern (MTN) Blot (Clonetech) containing each poly A^+ RNA from human (a) heart, (b) brain, (c) placenta, (d) lung, (e) liver, (f)

skeletal muscle, (g) kidney and (h) pancreas, which was prepared by: modification of the electric charge of a nylon membrane prior to transferring the RNA, the RNA transfer from a 1.2% formaldehyde denaturalized agarose gel which had been previously loaded with 2 μ g of the above each poly A^+ RNA and electrophoresed, and then followed by a fixation using UV irradiation.

Hybridization was carried out using the above-described probe solution and membrane, in accordance with the following procedures. First, the membrane was immersed in 2 x SSC for 5 minutes, then prehybridization was performed in 10ml of hybridization solution (5 x SSC, 10 x Denhardt's solution, 10 mM sodium phosphate buffer (pH 6.5), 50% formamide, 0.5%SDS, 0.1 mg/ml sermon sperm DNA), at 65℃ for 3 hours. Subsequently, the probe which had previously been boiled for 10 minutes and rapidly frozen with dry ice/ethanol for 5 minutes was diluted in the hybridization solution to be 1 μ g/ml, and 2 ml of thus diluted probe solution was used for hybridization at 65℃ for 18 hours.

Following hybridization, the membrane was washed by shaking: two times in 20 ml of 2 x SSC, 0.1%SDS at a room temperature for 5 minutes and then two times in 20 ml of 0.1 x SSC, 0.1%SDS at 68℃ for 15 minutes. Next, the membrane was washed two times with 50 ml of DIG buffer I at a room temperature for one minute in order to remove SDS, and was blocked in 50 ml of DIG buffer II' at a room temperature for one hour. Thereafter, the membrane was treated with 10 ml of alkaline phosphatase labeled anti-DIG antibody which was diluted to 5000-fold in DIG buffer I which contains 0.2% Tween20 for 30 minutes, followed by washing two times by shaking in 50 ml of DIG buffer I which contains 0.2% Tween20 at a room temperature for 20 minutes. After soaking the membrane twice in 10 ml of DIG buffer III at a room temperature for 3 minutes, it was placed in a hybridization bag, and

CSPD which was diluted to 100-fold in DIG buffer III was added thereto so that the solution can spread over the membrane. Subsequently, the membrane was exposed to Instant Film 612 (Polaroid).

As a consequence, it was revealed that mRNA of the collectin of the present invention having 1.2 kb and 3.8 kb in size has been expressed in liver (lane e) and in placenta (lane c), with more amount being expressed in liver while less but certain amount being expressed in placenta as shown in Figure 9.

Example 7: Genomic Southern Analysis of the Novel Collectin in Various Animals

In order to elucidate conservation of the collectin gene of the present invention between other species of animals, analysis by genomic Southern hybridization was performed.

Hybridization probe employed for this analysis was the DIG labeled DNA probe corresponding to the cDNA sequence of ORF of the novel collectin as obtained in the above Example 4, which was labeled using the above-described PCR DIG Probe Synthesis Kit (Boehringer Mannheim), while the analyzed membrane was ZOO-BLOT (Clonetech). This membrane contains each genomic DNA obtained from (a) human placenta, (b) Rhesus monkey kidney, (c) Sprague-Dawley rat kidney, (d) Balb/c mouse kidney, (e) canine kidney, (f) bovine kidney, (g) rabbit kidney and (h) chicken liver, which was prepared by modification of the electric charge of a nylon membrane prior to transferring the genomic DNA, the DNA transfer from an agarose gel which had been previously loaded with 4 μ g of the above each genomic DNA which were digested with restriction enzyme EcoRI, and electrophoresed, then finally followed by a fixation using UV irradiation.

Hybridization was carried out using the above-described probe and membrane, in accordance with the following procedures. First, the membrane was immersed in 2 x SSC for 5 minutes, then prehybridization was performed in 10ml of ExpressHyb Hybridization Solution at 65℃ for 30 minutes. Subsequently, the probe which had previously been frozen as described above was diluted in the ExpressHyb Hybridization Solution to be 10 ng/ml, and 2 ml of thus diluted probe solution was used for hybridization at 65℃ for one hour.

Following hybridization, the membrane was washed by shaking: two times in 20 ml of 2 x SSC, 0.1%SDS at a room temperature for 5 minutes and then two times in 20 ml of 0.2 x SSC, 0.1%SDS at 68℃ for 15 minutes. Next, the membrane was washed two times with DIG buffer I at a room temperature for one minute in order to remove SDS, and was blocked in 50 ml of DIG buffer II' at a room temperature for one hour. Thereafter, the membrane was treated with 10 ml of alkaline phosphatase labeled anti-DIG antibody which was diluted to 5000-fold in DIG buffer I which contains 0.2% Tween20 for 30 minutes followed by washing two times with shaking in 50 ml of DIG buffer I which contains 0.2% Tween20 at a room temperature for 20 minutes. After soaking the membrane twice in 10 ml of DIG buffer III at a room temperature for 3 minutes, it was placed in a hybridization bag, and CSPD which was diluted to 100-fold in DIG buffer III was added thereto so that the solution can spread over the membrane. Subsequently, the membrane was exposed to Instant Film 57.

The result of this analysis is shown in Figure 10, wherein signals of DNA can been found in all lanes except for the lane h which was loaded with DNA from chicken. Therefore, it was illustrated that the collectin gene of the present invention has been conserved between the mammalian species.

Example 8: Genetic Analysis of the Novel Collectin

To elucidate the genetic positional relevance of the present collectin between the known collectins, analysis was performed based on the DNA sequence as obtained, and a phylogenetic tree was created.

The collectins selected as subjects for analysis were: human MBP (Mannan- Binding Protein), human SP-A (Surfactant Protein A), rat MBP-A, rat MBP-C, rat SP-D, mouse MBP-A, mouse MBP-C, rabbit MBP, monkey MBP-A, monkey MBP-C, bovine SP-D, bovine MBP, bovine conglutinin (bKg), and bovine collectin 43 (CL43). Each of the amino acid sequence was retrieved from GenBank data base, then using the regions containing lectin domains from the obtained data, multiple alignment was produced by clustalw method. Thereby, a phylogenetic tree was created using N-J method with Phylip Version 3.57c package program.

Consequently, as shown in Figure 11, although SP-D, bovine collectin 43 and bovine conglutinin have constituted single cluster, additionally MBP and SP-A have respectively constituted separate clusters, while the collectin gene of the present invention has not belonged to any of these clusters. Accordingly, it was speculated that the collectin of the present invention may constitute a distinct cluster which is genetically diverse from those of the collectins reported heretofore.

[Industrial Applicability]

As set forth above, a novel collectin gene and protein having characteristic structures of the collectins, which are different from the collectins reported so far, are provided by the present invention. Such protein is expected to exhibit physiological activities such as anti-bacterial, anti-viral activity, especially in human body, thereby medicinal applications, as well as tools for investigating mechanisms of biological defense systems may be

provided.